

Application of bacteriophages for detection and control of foodborne pathogens

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Abstract The incidence of foodborne infectious diseases is stable or has even increased in many countries. Consequently, our awareness regarding hygiene measures in food production has also increased dramatically over the last decades. However, even today's modern production techniques and intensive food-monitoring programs have not been able to effectively control the problem. At the same time, increased production volumes are distributed to more consumers, and if contaminated, potentially cause mass epidemics. Accordingly, research directed to improve food safety has also been taken forward, also exploring novel methods and technologies. Such an approach is represented by the use of bacteriophage for specific killing of unwanted bacteria. The extreme specificity of phages renders them ideal candidates for applications designed to increase food safety during the production process. Phages are the natural enemies of bacteria, and can be used for biocontrol of bacteria without interfering with the natural microflora or the cultures in fermented products. Moreover, phages or phage-derived proteins can also be used to detect the presence of unwanted pathogens in food or the production environments, which allows quick and specific identification of viable cells. This review intends to briefly summarize and explain the principles and current standing of these approaches.

Keywords Bacteriophage · Pathogens · Listeria · Salmonella · E. coli · Mycobacterium

Introduction

Statistical data on foodborne disease is fragmented, focusing either on particular countries or on particular pathogens only. This situation is even worse with no official records existing, as is the case with listeriosis in Portugal (Almeida et al. 2006). Nonetheless, the number of cases of several foodborne diseases has been rising continuously. When first described in 1983, *Escherichia coli* O157:H7 was regarded as a rare serotype (Riley et al. 1983), while today it is one of the major causes of foodborne disease in industrial countries. Outbreaks of disease caused by foodborne pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, *Listeria*, and others have an enormous impact on public health.

Bacteriophages are viruses infecting bacteria; they are obligate intracellular parasites and lack their own metabolism. Phages are extremely host-specific, able only to infect specific species or even strains, with a few exceptions such as *Listeria* phage A511, which can infect and kill bacteria within an entire genus (Zink and Loessner 1992). The available data clearly indicate that phages can contribute to lowering the incidence of such outbreaks, both by facilitating rapid detection and as a biocontrol agent.

The majority of phages described to date belong to the order *Caudovirales*, tailed phages with isometric heads containing double-stranded DNA. The specificity of these phages is partly mediated by tail-associated proteins that distinctively recognize surface molecules of susceptible bacteria. Temperate phages can integrate themselves into the host genome after infection. This can lead to an altered

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phenotype of the host and in some cases, even increase bacterial pathogenicity (Eklund et al. 1971; O'Brien et al. 1984; Figueroa-Bossi et al. 2001). Therefore, temperate phages are generally considered unsuitable for the purpose of eradicating unwanted bacteria. Virulent phages are unable to integrate into their host genome, with successful infection always resulting in the death of their host, and generally have broader host ranges. Generally speaking these phages are ideally suited for detection purposes, and, for biocontrol approaches, virulent phages are the only option.

Bacteriophages for detection of bacterial pathogens

Phage typing

Phage typing is a popular tool to differentiate bacterial isolates, and is used in epidemiological studies with the aim of identifying and characterizing outbreak-associated strains. Individual phages may recognize a multitude of different surface-associated molecules (Eriksson and Lindberg 1977; Estrela et al. 1991; Joys 1965; Schwartz 1983; Sun and Webster 1987; Hung et al. 2002), and after recognition and injection of the viral DNA, target cells may still fail to produce progeny phage (Sturino and Klaenhammer 2004). Although more sophisticated systems for differentiation are available, such as ribotyping, random amplified polymorphic DNA-PCR fingerprinting, or pulsed field gel electrophoresis of enzyme-digested DNA, the variable sensitivity to a set of bacteriophages (phage typing) remains a useful method because of its speed, relative simplicity, and cost-effectiveness. Studies on enterohemorrhagic *E. coli* (EHEC) and *Campylobacter* showed that phage typing can be highly useful, especially because any one typing method alone fails to produce all the relevant data pertaining to epidemiological relatedness (Grif et al. 1998; Hopkins et al. 2004). Various phage typing schemes exist for all common foodborne pathogens such as *Salmonella*, *Campylobacter*,

E. coli, and *Listeria* (Scholtens 1962; Majtanova and Majtan 2006; Hopkins et al. 2004; Nicolle et al. 1952; Grif et al. 1998; Loessner 1991; Gasanov et al. 2005).

Rapid detection methods

Detection of pathogens using phages may not always provide the sensitivity of a PCR approach; it allows the distinction of living bacteria and is much more rapid than traditional cultivation techniques. While reverse transcription-based PCR assays also allow the distinction between living and dead bacteria, they are not applicable for routine diagnostics of a diverse range of foods. In recent years, different strategies to employ phages for detection have been put forward (Table 1). The specific and rapid detection of bacteria by measuring the activity of a reporter gene carried by a phage and expressed only after infection was first reported by Ulitzur and Kuhn (1987). Expression of bacterial luciferase genes (*lux*) from *Vibrio fischerii* cloned into lambda vector detected as little as ten *E. coli* cells in milk within 1 h. Luciferase genes have the enormous advantage that background photon emission should be absent from food samples, and luminescence thus always reflects the presence of viable target bacteria. Phage lambda vectors allow direct cloning of large DNA fragments, but, being a temperate phage with a relatively narrow host range, application is limited. Unfortunately, for most other phages, direct cloning does not exist. Insertion mutagenesis, using transposons carrying the desired reporter gene, represents an alternative. When phage genome sequences are available, reporter genes can be inserted via homologous recombination from a plasmid. While these techniques may work with most phages, the maximum genome length that can be packaged into phage head limits the size of additional information that can be incorporated. For this reason, only the *lux* genes specifying the luciferase enzyme have been introduced into most luciferase reporter phages (LRPs), necessitating the external addition of the substrate.

Table 1 Different approaches harnessing phage for detection of bacterial pathogens

Type of phage/detection method	Target organism	References
LRP (<i>lux</i> , <i>luc</i>)	<i>E. coli</i> , <i>Salmonella</i> , <i>Enterobacteriaceae</i> , <i>Mycobacteria</i> , <i>Listeria</i>	Ulitzur and Kuhn 1987, 2000; Waddell and Poppe 2000; Stewart et al. 1989; Kuhn et al. 2002; Kodikara et al. 1991; Riska et al. 1999; Sarkis et al. 1995; Banaiee et al. 2003; Loessner et al. 1996, 1997
Ice nucleation reporter phage	<i>Salmonella</i>	Wolber and Green 1990; Kuhn et al. 2002
AK release through lysis by native phages ^a	<i>Salmonella</i> , <i>E. coli</i> O157:H7, <i>Listeria</i>	Blasco et al. 1998; Wu et al. 2001
Labeled phages (GFP, YOYO-1) ^a	<i>E. coli</i> O157:H7	Goodridge et al. 1999; Oda et al. 2004; Tanji et al. 2004
<i>lacZ</i> reporter phage ^a	<i>E. coli</i> O157:H7	L. Goodridge (personal communication)
Plaque formation (semiquantitative) ^{a,b}	<i>Salmonella</i>	Favrin et al. 2001

^a Prior IMS required for specificity

^b Requires addition of a virucide to inactivate free phage particles

Construction of variety of LRPs using the methods mentioned above have been reported by Ulitzur and Kuhn (2000). However, little information exists on the properties of these phages, with the exception of a phage P22-based *Salmonella* LRP, the efficacy of which was studied and demonstrated in two separate studies (Stewart et al. 1989; Turpin et al. 1993). Using another *Enterobacteriaceae*-specific LRP, detection of approximately 10^4 cells g^{-1} was possible after 50 min, and detection of 10 cells g^{-1} after only 4 h of enrichment (Kodikara et al. 1991). Although the studies based on P22 both showed that small numbers of *Salmonella* cells could be detected, this phage is temperate and has a limited host range. In 2000, an O157:H7 specific LRP was constructed, based on the temperate phage Φ V10 (Waddell and Poppe 2000). Once again, although able to detect small numbers of target cells, the LRP constructed was based upon a temperate phage with a limited host range of 67% of O157H:7 isolates.

Construction of a broad host range *Listeria* LRP based on the virulent and genus-specific phage A511 was constructed, infecting approximately of 95% of clinically relevant *Listeria monocytogenes* serovars (Loessner et al. 1996). Introduction of the *luxAB* gene fusion cassette into A511::*luxAB* was achieved via homologous recombination, placing the *Vibrio harveyi luxAB* genes under transcriptional control of the phage major capsid promoter Cps. The efficacy of A511::*luxAB* was demonstrated using both spiked and naturally contaminated food samples. In most cases, very low contamination levels of a single cell per gram could be detected within 24 h (Loessner et al. 1997).

Conventional protocols for detection of pathogens in food require a minimum of 48–72 h to obtain preliminary results. A broad host range LRP for *Salmonella*, based on the virulent phage Felix-O1, was reported by Kuhn et al. (2002). After attempts to insert the *luxAB* genes into the phage genome via transposon-mediated insertion, part of the Felix-O1 genome was eventually replaced with the luciferase genes. This resulted in a defective, nonreplicating phage, the propagation of which was possible only using a strain engineered to provide the missing genes in *trans*. As a drawback, the authors found that the bioluminescence signals of the resulting LRP preparation were inconsistent. This problem was not encountered when using the ice nucleation gene (*ina*) as a reporter in the same phage background setting. Use of *ina* as a reporter in phage detection was first reported in 1990, with phage P22 (Wolber and Green 1990). The *Ina* protein encoded by the phage and expressed by the infected host cells allowed detection of very low contamination levels (2 cells ml^{-1}) (Wolber 1993).

Several LRPs are available for *Mycobacterium tuberculosis* (Sarkis et al. 1995; Riska et al. 1999; Banaiee et al. 2003). Clearly, the relative speed of phage-based assays is especially useful in measures to detect slow-growing organisms.

While the *ina* and *lux* genes share the advantage that background noise originating from food samples are unlikely to occur and interfere with detection, assays based on enzymes such as *lacZ* may be even simpler and require less expensive equipment. Toward this end, a reporter phage for detection of O157:H7 has been constructed, by introducing *lacZ* into a T4 phage by transposon mutagenesis (L. Goodridge, personal communication). However, the occurrence of natural galactosidase in samples and the broad interspecies host range of T4-like phages necessitate additional steps before phage infection, such as immunomagnetic separation (IMS) with O157:H7-specific antibodies for pre-separation of target cells.

IMS also provides the basis for pathogen detection based on bacterial enzymes liberated from the captured cells after phage-induced lysis (Blasco et al. 1998; Wu et al. 2001). After preincubation, target cells are concentrated by IMS, and specific bacteriophages are then used for infection and lysis of host cells, resulting in the release of adenylate kinase (AK). Upon addition of ADP, AK converts the substrate to ATP, which can be detected by firefly luciferase.

The usefulness of phage-based cell wall recognition proteins for magnetic capture has recently been described (Kretzer et al. 2007). This system is based upon the unique properties of phage peptidoglycan hydrolases. These endolysins feature a two-domain functional structure, where enzymatically active domains are linked to cell-wall-binding domains (CBDs) (Korndoerfer et al. 2006). The CBDs are highly specific for recognition and binding to the target cells surfaces, and feature an unusual high affinity (Loessner et al. 2002). Paramagnetic beads coated with the CBD molecules were shown to perform much better than commercially available antibody-based beads, both with respect to sensitivity and percent recovery (Kretzer et al. 2007). Another potential strategy is the identification of phage-tail-associated recognition proteins, which might prove useful for immobilization of gram-negative cells.

Incorporation of reporter genes may not always be possible due to limited genome capacity and a lack of nonessential regions in the used phages. An alternative to circumvent these limitations is direct labeling of phage particles.

By labeling phage DNA with a fluorescent stain, Goodridge et al. (1999) were able to quickly detect *E. coli* O157:H7 cells in a variety of foods after preincubation and IMS. The phage LG1 is specific to *E. coli* O157:H7, and was used essentially like a labeled antibody. More recently, two reports describe the construction of GFP-labeled reporter phages for direct detection of *E. coli* (Oda et al. 2004; Tanji et al. 2004). In the first study phage PPO1, specific to *E. coli* O157:H7, was modified to incorporate GFP in its capsid, allowing external decoration of target cells, also including injured cells.

Based on plaque formation, a semiquantitative phage-based assay type has been described (for review, see Rees and Loessner 2005). In the case of *Salmonella* (Favrin et al. 2001), bacteria are first captured by IMS, then infected with phage, followed by inactivation of remaining extracellular phage with a virucide. Immediately thereafter, the infected cells mixed with phage indicator bacteria and plated on soft agar double-layer plates. The infected target cells will then lyse, release progeny phages, which results in plaque formation.

Bacteriophages for control of pathogens in food

The concept of combating pathogens by means of phages is obvious, and was proposed shortly after the discovery of phages approximately 90 years ago. Unfortunately, the discovery of antibiotics basically eliminated research on phage therapy. Today, the increasing problem of antibiotic resistance rekindled the interest in phage therapy, but only very recently was the concept extended to the field of food safety. Two important concerns need to be addressed: Are the effects of phages harmless upon consumption, and how can phage resistance be dealt with? Phages are highly specific in recognition of host cells, and no adverse effects on commensal bacteria should be expected by the oral consumption of phages targeted at pathogens. The ubiquitous nature of phages and the high numbers of phages found in natural habitats such as seawater, fresh water, soil, plant material, and even food (Kennedy and Bitton 1987) as well as the ease with which phages can be isolated from human feces corroborate this assumption. An oral toxicity study in rats receiving high doses of *Listeria* phage P100 did not reveal any side effects (Carlton et al. 2005). A study in humans with *E. coli*-specific phages also indicated that phages are safe for oral administration (Bruttin and Brüssow 2005). While bacteria have developed specialized phage defense mechanisms, phages also continuously adapt to these altered host systems. In this context, it is interesting to note that spontaneous mutations conferring phage resistance may actually have deleterious effects on these bacteria, and not necessarily confer an evolutionary advantage in the absence of phages, a phenomenon that has been observed during experimental phage treatment of *E. coli*-O157:H7-contaminated beef (O'Flynn et al. 2004). Furthermore, technical measures such as the alternating use of different phages, either in a cocktail or in consecutive treatments, may also reduce the frequency of resistance.

Several current strategies to combat livestock-associated pathogens such as toxinogenic *E. coli*, *Campylobacter*, and also *Salmonella* are direct extensions of "classical" phage therapy approaches, in so far as they focus on targeting bacteria in the animals before slaughter (Sheng et al. 2006; Wagenaar et al. 2005; Fiorentin et al. 2005). On the other

hand, food contamination with, e.g., *L. monocytogenes*, is more likely to occur during food processing, which consequently is the most reasonable time point for phage biocontrol of this pathogen. In this context, the US FDA has recently approved use of anti-*Listeria* phages food additives, and conferred the generally recognized as safe status to another anti-*Listeria* phage.

Bacteriophage treatment of toxinogenic *E. coli* and *Enterobacter*

The EHEC *E. coli* O157:H7 can cause severe and sometimes fatal hemorrhagic diarrhea in humans (O'Brien et al. 1993). Ruminants carrying these bacteria are not significantly affected, and form the principal reservoir for this strain. Contamination of animal products can occur during milking or slaughter. Two studies report experimental phage treatment of sheep (Bach et al. 2003; Raya et al. 2006). While Bach et al. observed clearance of bacteria due to phage DC22 application in an artificial rumen system, oral administration of phages to lambs did not reduce fecal shedding of target bacteria. This may be the result of insufficient numbers of phages reaching the intestine, however, Raya et al. were able to observe a 2–3 log₁₀ reduction in intestinal EHEC O157:H7 after oral administration of phage CEV1. Other authors (Sheng et al. 2006) treated mice, sheep, and cattle with phages KH1 and SH1. Oral administration of KH1 to sheep did not reduce numbers of intestinal target bacteria. Oral application of either SH1 or a combination of KH1 and SH1 in mice resulted in complete eradication of target bacteria. To exclude loss of phages during passage to the intestine, phages were administered rectally to steers. It is interesting to note that one of the five treated animals remained culture negative for the target bacterium, and significantly lower cell numbers were observed in the remaining four animals when compared to the control group.

As alternative to such in vivo treatments, phages can be applied or mixed directly onto or into the food product. O'Flynn and coworkers used a cocktail of three different phages to treat beef contaminated with 10³ CFU g⁻¹ *E. coli* O157:H7. In seven out of nine samples, no viable cells could be retrieved after storage at 37°C, while in the remaining two samples counts were below 10 CFU g⁻¹ (O'Flynn et al. 2004).

A recent study addressed the problem of *Enterobacter sakazakii* growing in reconstituted infant formula milk (Kim et al. 2007), employing newly isolated phages against this infectious pathogen. It is interesting to note that the T4-like phage ESP 732-1 was able to effectively suppress the growth of the organism in prepared infant formula, both at 24 and 37°C. The killing effect was dose-dependent, with the highest phage concentration (10⁹ pfu/ml) being most effective and able to completely eradicate the target organisms.

Bacteriophage treatment of *Campylobacter*

Oral infection with *Campylobacter* has become the most common cause of foodborne disease in industrialized nations. Two species, *Campylobacter jejuni* and *Campylobacter coli* are of particular concern. The cells are microaerophilic, have an optimum growth temperature of approx. 41°C, and are often associated with poultry. The infective dose of only 400–500 cells is thought to be the major reason underlying the widespread infection (Moore et al. 2005).

In an attempt to understand the natural ecology of *Campylobacter* and their phages, Atterbury et al. (2005) conducted a study that showed a negative correlation between the number of bacteria and presence of phages in barn-held broiler chickens. Other recent studies have focused on phage-based eradication of *Campylobacter* in broiler flocks because this would lower the risk of cross-contamination during slaughter (Loc Carrillo et al. 2005; Wagenaar et al. 2005). Here, significant decreases in *Campylobacter* counts recovered from cecal contents were observed. Wagenaar et al. assessed both curative treatment of existing *Campylobacter* colonization, and pretreatment of hatched chickens to prevent colonization. However, the results were only partly successful: After an initial reduction in bacterial numbers in the curative treatment, bacterial levels stabilized slightly below levels of the untreated controls. In the prophylactic trials, a delay in colonization was observed. A potential solution might be to administer a pool of different phages, in higher concentration, in a form to be delivered to the lower GIT compartments. Phage application shortly before slaughter may also contribute to more favorable results. With respect to application of phage directly on food, Goode et al. (2003) were able to achieve a 95% reduction in *C. jejuni* counts on artificially contaminated chicken skin.

Bacteriophage treatment of *Salmonella*

Salmonella remains one of the principal causes of food-related illness. Phage biocontrol measures have been reported both in vivo and on food. Goode et al. (2003) observed eradication of phage-susceptible *Salmonella* strains. Moreover, these authors also report that phage-resistant strains were affected at higher phage concentrations. Infection by more than one phage can kill cells directly, and leads to collapse of the membrane potential and subsequent cell death (Tarahovsky et al. 1994).

An earlier study examined the effect of a phage cocktail on low-acid fruits (Leverentz et al. 2001). A significant reduction of target bacteria was observed on melon but not on apple. This corresponded to the number of viable phages on the two foodstuffs. While phage numbers remained

relatively stable on melon, a rapid decline of infective phage particles was observed on apples.

Whichard et al. (2003) tested the broad host range *Salmonella* phage Felix-O1 in biocontrol experiments with *Salmonella typhimurium* on sausages, and reported a 2 log₁₀ reduction of viable cells. In another study, incorporation of phage SJ2 into the starter culture and artificial contamination with *Salmonella* during cheddar production resulted in the absence of viable cells after several months of storage, whereas high numbers of target bacteria were isolated from nontreated controls (Modi et al. 2001).

Different studies describe attempts to reduce *Salmonella* directly in broiler chickens (Fiorentin et al. 2005). A high dose of phage was administered orally, resulting in a significant reduction of bacterial counts compared to controls. Although the reduction in viable cells persisted for more than 3 weeks, no complete eradication of *Salmonella* could be achieved.

Bacteriophage to control *Listeria* contamination

Of the foodborne pathogens of interest, infection by *L. monocytogenes* may have the lowest incidence. However, because of the high mortality rate of up to 30%, its ability to survive a wide range of environmental conditions and its ability to multiply at refrigeration temperatures, *Listeria* is considered an important pathogen (Farber and Peterkin 1991). Because of its ubiquitous nature, various contamination routes are conceivable, and recontamination is prevalent. Therefore, phage treatment during final processing and or packaging appears the most reasonable option. A *Listeria* phage cocktail was tested on fresh cut produce, alone and in combination with a bacteriocin (Leverentz et al. 2003, 2004). Similar to what was observed with *Salmonella* phages, phages showed reduced stability and efficacy on apple slices. It is interesting to note that phages alone (reduction by up to 4 log₁₀) performed better than the bacteriocin alone and the combined application showed an additive effect. However, complete eradication of *Listeria* was not observed in these experiments. In a different study, which also evaluated in vivo feeding toxicity and addressed the issue of potential allergenicity by an in silico approach, the effect of the broad host range, virulent phage P100 on growth of *Listeria* in soft cheese was studied. Complete eradication of target cells was achieved, depending on dosage and treatment schedule (Carlton et al. 2005). In a recent comprehensive study, involving a large variety of different food of plant and animal origin, virulent phages A511 and P100 were able to achieve reduction in cell counts by 2 to approx. 4 log₁₀ units, confirming the enormous potential and versatility of phages as biocontrol agents against *Listeria* (S. Guenther and M.J. Loessner, submitted for publication).

Conclusions

While not likely to replace other existing measures for detection of pathogens, the advantages of speed combined with the ability to differentiate between living and dead cells, phage-based assays are highly suitable to augment more traditional methods. With increasing knowledge and technical possibilities, it is likely that the prevalence and acceptance of phage-based detection methods will rise and commercial applications become more widely available.

Phages are a logical candidates for the fight against undesired bacteria. There is no doubt that their application in biocontrol of pathogens will benefit from the very active research in this field. It is safe to assume that nontransducing, virulent phages is harmless to humans, and can make a valuable contribution to food safety and public health.

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